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REVIEW

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Evolution of the metal hyperaccumulation and hypertolerance traits

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Abstract

To succeed in life, living organisms have to adapt to the environmental issues to which they are subjected. Some plants, defined as *hyperaccumulators*, have adapted to metalliferous environments, acquiring the ability to tolerate and accommodate high amounts of toxic metal into their shoot, without showing symptoms of toxicity. The determinants for these traits and their mode of action have long been the subject of research, whose attention lately moved to the evolution of the hypertolerance and hyperaccumulation traits. Genetic evidence indicates that the evolution of both traits includes significant evolutionary events that result in species-wide tolerant and accumulating backgrounds. Different edaphic environments are responsible for subsequent refinement, by local adaptive processes, leading to specific strategies and various degrees of hypertolerance and hyperaccumulation, which characterize metallicolous from non-metallicolous ecotypes belonging to the same genetic unit. In this review, we overview the most updated concepts regarding the evolution of hyperaccumulation and hypertolerance, highlighting also the ecological context concerning the plant populations displaying this fascinating phenomenon.

KEYWORDS

Brassicaceae, evolution of abiotic stress resistance, heavy metals, metalloids

1 | INTRODUCTION

Plants must rapidly evolve to adapt to environmental cues. Heavy metal-rich soils, either naturally enriched or those contaminated by recent anthropogenic activities, constitute a serious challenge to plant survival.

Some plant species can live and reproduce on metalliferous soils and are characterized by high tolerance (i.e. hypertolerance) against normally toxic amounts of heavy metals in the growth substrate. In this context, hypertolerance can be displayed by plants through two opposite mechanisms. On the one hand, some plants can exclude toxic metals from absorption, thereby limiting the entry and the root-to-shoot translocation of the metals to physiological concentrations (Verbruggen, Hermans, & Schat, 2009). On the other hand, other species have evolved a peculiar ability to accumulate two to four times higher levels of metal (or metalloids) in their aerial parts than those found in non-accumulating plants in the same environment (Reeves & Baker, 2000; Van der Ent, Baker, Reeves, Pollard, & Schat, 2013). These species are defined as hyperaccumulators. The differences in hyperaccumulation and exclusion strategies of hypertolerant plants are shown in Figure 1.

The first description of a plant able to gather extremely high levels of metals dates back to 1948, when *Alyssum bertolonii* plants, growing in the Tuscan region of Italy, were reported to accumulate a Ni content of more than 1 mg/g of dry weight in the shoot (Minguzzi & Vergnano, 1948). Only in 1976 was the term *hyperaccumulator* coined to indicate plants able to accumulate high metal concentrations in the shoot without showing symptoms of toxicity, demonstrating association between metal hypertolerance and the ability to detoxify the metals stored (Jaffrè, Brooks, Lee, & Reeves, 1976). The threshold values for defining a plant as a hyperaccumulator were subsequently established for each different

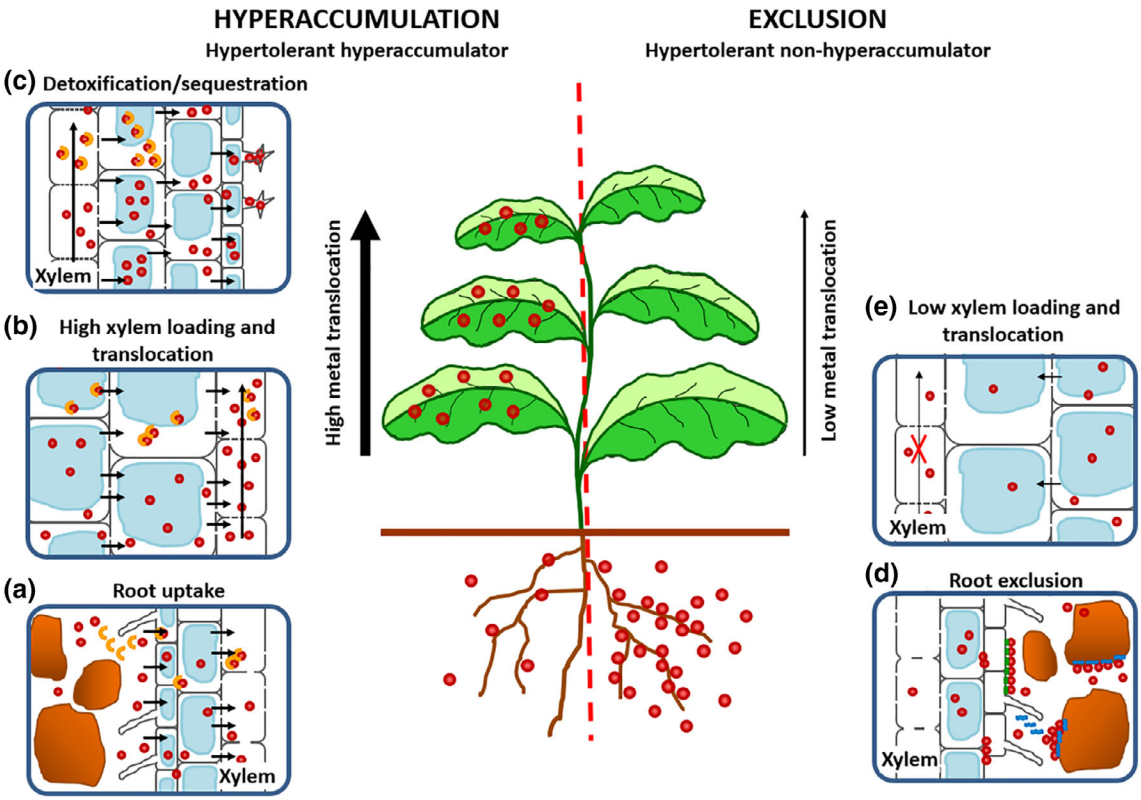

FIGURE 1 Main processes involved in hypertolerance and metal distribution in a hyperaccumulator plant (on the left side) and in an excluder non-accumulator plant (on the right side). Mechanisms involved in metal hyperaccumulation include: (a) enhanced metal uptake in roots and a reduced accumulation in root vacuoles, (b) efficient xylem loading and root-to-shoot translocation and (c) a strong metal chelation in the cytosol in the shoot and/or compartmentalization in leaf vacuoles. Mechanisms involved in metal exclusion include: (d) reduced metal uptake in roots and chelation in soil by root exudates, and (e) inhibition of xylem loading and translocation to the shoot (Metal and metalloids are indicated by red dots. Chelating molecules are indicated by yellow, blue and green symbols) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Hyperaccumulation thresholds for the most relevant metals and numbers of hyperaccumulator plant families, genera and species discovered to date, as reported in the Global Hyperaccumulator Database (March 2020; http://hyperaccumulators.smi.uq.edu.au/collection/, Reeves et al., 2017)

Element	Threshold for hyperaccumulators (mg/kg dw)	Families (No.)	Genera (No.)	Species (No.)
Arsenic (As)	>1,000	1	2	5
Cadmium (Cd)	>100	7	9	43
Cobalt (Co)	>300	18	33	43
Copper (Cu)	>300	20	44	53
Chromium (Cr)	>1,000	1	1	1
Lead (Pb)	>1,000	7	9	9
Manganese (Mn)	>10,000	16	24	42
Nickel (Ni)	>1,000	54	131	532
Selenium (Se)	>100	7	15	41
Thallium (Tl)	>100	1	2	2
Zinc (Zn)	>3,000	9	12	21

metal, metalloid and semimetal (Table 1). According to recently suggested criteria, hyperaccumulators are plants that, when growing on their natural sites, are able to accumulate >10 mg/g (1%) Mn, >3 mg/g Zn (0.3%), >1 mg/g (0.1%) As, Cr, Ni or Pb, >0.3 mg/g (0.03%) Co or Cu, and >0.1 mg/g (0.01%) Cd, Se or Tl in the aerial organs (Reeves et al., 2017).

These plants accumulate metals in their above ground tissues, thereby removing these elements from roots (Jaffrè et al., 1976; Maestri, Marmiroli, Visioli, & Marmiroli, 2010). This is due to the enhancement of some physiological processes, such as root metal uptake, symplast mobility, xylem loading and unloading, and is associated with a greater ability to counteract the toxicity of the metal stored in the aerial parts, especially leaves, by chelation and vacuolar sequestration, thus implying hypertolerance (Krämer, 2010; Verbruggen et al., 2009). Genetic determinants for hyperaccumulation and hypertolerance do not fall in the same genetic *loci* and, indeed, these phenomena should be considered as independent, as explained in more detail in the following paragraphs.

2 | METAL HYPERACCUMULATION AND HYPERACCUMULATOR PLANT SPECIES

Hyperaccumulation is an extreme evolutionary trait that has appeared independently many times. It was reported in a wide range of species belonging to distantly related families, including about 0.2% of all known angiosperms (Baker, McGrath, Reeves, & Smith, 2000; Krämer, 2010). As of March 2020, in consideration of recent discoveries and new accumulation thresholds, the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/, Reeves et al., 2017) includes 759 species, some of which can store more than one element. The numbers of hyperaccumulator species, genera and families discovered for each element are reported in Table 1. Of the 82 families of hyperaccumulator plants, Brassicaceae (in particular the genera *Noccaea* and *Alyssum*) and Phyllanthaceae are the most highly represented, with 104 and 130 species, respectively. Given that many as yet unidentified hyperaccumulator plants may occur in nature, this number is certain to increase as new discoveries on this kind of plants are made.

Such plants are mainly biennial or short-lived perennial herbs, shrubs or small trees that are endemic in metal-rich soils in both tropical and temperate zones worldwide (Baker & Brooks, 1989; Bhatia, Baker, Walsh, & Midmore, 2005). Their distribution often appears to be confined to metalliferous or metal-contaminated soils, where they become dominant over other species, existing as almost pure populations: in this case, they behave as *strict metallophytes*. For these species, a high metal concentration in the soil is essential for normal growth (Rascio & Navari-Izzo, 2011). On the other hand, some *facultative metallophytes* or *pseudo-metallophytes* can also survive on non-metalliferous soil but they barely compete with non-tolerant species in such a non-selective condition. This is probably due to the high costs in terms of energy and the allocation of resources required to counter metal toxic effects and support element accumulation (Maestri et al., 2010).

In some species, hyperaccumulation is a constitutive trait found in all populations, as in the case of the Zn hyperaccumulators—*Noccaea caerulescens* (formerly, *Thlaspi caerulescens*; Escarré et al., 2000) and *Arabidopsis halleri* (Bert, Macnair, De Laguerie, Saumitou-Laprade, & Petit, 2000). Both are close, wild relatives of *Arabidopsis thaliana*, which offers a wide variety of genomic, transcriptomic and proteomic data, as well as genomic tools and molecular markers (Clauss, Cobban, & Mitchell-Olds, 2002). This allows for easier analysis and a deeper investigation of the trait in these species. Indeed, *A. halleri* and *N. caerulescens* share with *A. thaliana* an average nucleotide identity within coding regions of 94 and 88%, respectively (Krämer, 2010), and a consistent synteny in the genomic regions, facilitating quantitative trait loci (QTL) mapping and the identification of candidate genes (Pauwels, Willems, Roosens, Frérot, & Saumitou-Laprade, 2008). As stated above, all characterized populations of both species hyperaccumulate Zn when grown on non-metalliferous soils. Indeed, some populations of *N. caerulescens* accumulate high Zn concentrations (more than 1% in leaves) irrespective of the Zn concentration of the substrate (Reeves, Schwartz, Morel, & Edmondson, 2001). A similar ubiquitous hyperaccumulation ability was observed in *A. halleri* populations, native to non-metalliferous soils (Stein et al., 2017). Differently from constitutive hyperaccumulating species, as *N. caerulescens* and *A. halleri*, other species, such as *Sedum alfredii* and *Senecio coronatus*, include both hyperaccumulating and non-accumulating populations (Lu et al., 2008; Mesjasz-Przybyłowicz, Barnabas, & Przybyłowicz, 2007). In *S. coronatus*, hyperaccumulation ability does not correlate with the edaphic context of the different populations, since non-accumulators were also found in metalliferous soils (Meier et al., 2018).

Access to a variety of behaviours among different species and populations has allowed an extensive study to be conducted into both hypertolerance and hyperaccumulation, and their genetic independence. For instance, the analysis of F_2 populations, derived from the cross between *A. halleri* (tolerant) and close *Arabidopsis lyrata* subsp. *petraea* (metal sensitive, non-tolerant), reveal that Zn tolerance and hyperaccumulation are genetically independent traits (Macnair, Bert, Huitson, Saumitou-Laprade, & Petit, 1999). In addition, when a population of F_1 plants, derived from *A. halleri* × *A. lyrata*, were backcrossed to *A. lyrata* subsp. *petraea*, the co-segregation analysis supported the idea that Cd tolerance and accumulation are also independent traits. Interestingly, Cd and Zn tolerance co-segregate, suggesting a certain degree of pleiotropic control (Bert et al., 2003). More recent works performed on two geographically distant European *A. halleri* metallicolous populations, PL22 (from southern Poland) and I16 (from northern Italy), have similarly found high Zn and Cd tolerance but different accumulation capacity. PL22 hyperaccumulates Cd, whereas I16 stores significantly lower Cd levels than the hyperaccumulation threshold, resembling the excluder strategy (Corso et al., 2018).

Transcriptome analysis indicates that differences in gene expression are correlated with the genetic unit to which they belong rather than Cd treatment. In addition, flavonoid-related genes, which play a role in metal and ROS detoxification, were found highly expressed constitutively in PL22 with a consequently high accumulation of flavonoids in the shoots (Corso et al., 2018). Metallicolous populations of different genetic units displayed the evolution of diverse strategies for Cd uptake, transport and detoxification, headed by a different set of genes and metabolic pathway. Similarly, in an accompanying study

(Schvartzman et al., 2018), it was observed that these two metallicolous populations display similar Zn tolerance but different Zn accumulation. After Zn treatment for 2 weeks, plants belonging to PL22 exhibited hyperaccumulation levels, while their counterparts in the I16 population did not reach the shoot hyperaccumulation threshold (Schvartzman et al., 2018). Once again, the genetic unit was the principal driving force of the observed variation of gene expression and the Zn treatment had only a marginal effect on this variance.

In this context, regardless of the different Zn levels accumulated in shoots by the two populations, Zn treatment had almost no effect on gene expression in the shoots of either plant. This implies that hypertolerance is constitutive and 'controlled by the shoot' (i.e. Zn can accumulate in shoots without toxicity), whereas the accumulation itself is controlled by root processes (Corso et al., 2018; Schvartzman et al., 2018). Similar conclusions were also reported for Zn hyperaccumulation and hypertolerance in *N. caerulescens*. The analysis of plants obtained by reciprocal grafting between *N. caerulescens* and the Zn non-accumulator, *Thlaspi perfoliatum*, showed that shoots of plants with *N. caerulescens* as scion, on the rootstock of *T. perfoliatum*, did not hyperaccumulate Zn (Guimarães, Gustin, & Salt, 2009). Conversely, plants with a *T. perfoliatum* scion and the *N. caerulescens* rootstock showed Zn hyperaccumulation, although the plants suffered Zn toxicity symptoms. Thus, in *N. caerulescens*, Zn hypertolerance is driven by shoot processes while hyperaccumulation depends on the roots (Guimarães et al., 2009).

Intra-ecotypic genetic variations have been observed in several hyperaccumulating species (Pollard, Reeves, & Baker, 2014). For example, the Zn hyperaccumulation trait in *S. alfredii* (Yang et al., 2006) and the Cd hyperaccumulation in *A. halleri* (Bert et al., 2003) and *N. caerulescens* (Roosens, Verbruggen, Meerts, Ximenez-Embun, & Smith, 2003) have only been observed in some ecotypes belonging to these species. Similarly, in *S. coronatus* from South Africa, genetically-based differences in the inner cortical region and exodermis of the roots are responsible for a Ni concentration, that is, 35 times higher in Ni hyperaccumulating ecotypes than in non-accumulating individuals (Mesjasz-Przybyłowicz et al., 2007). Indeed, non-accumulating ecotypes showed increased suberin content in the exodermal Casparian bands in roots, thereby increasing their ability to work as apoplastic barriers (Mesjasz-Przybyłowicz et al., 2007). As mentioned above, once again the important role of the root physiology to the hyperaccumulation trait is evident.

Moreover, plastidial DNA analysis in *A. halleri* revealed that the population structure in northern Europe was related to geographical isolation rather than to Zn exposure. Therefore, metallicolous and non-metallicolous populations, living in close geographical areas, are genetically more similar than distant metallicolous populations (Pauwels, Saumitou-Laprade, Holl, Petit, & Bonnin, 2005), with the implication that the latter have evolved independently and, therefore, that the tolerance trait has also evolved in parallel, several times (Pauwels et al., 2008). As an interesting corollary, this validates the possibility that the evolution towards the same tolerance trait in different geographical areas implies convergent evolution, possibly through distinct evolutionary pathways (Alonso-Blanco et al., 2009).

3 | DISTRIBUTION OF METAL HYPERACCUMULATION IN PLANTAE

The majority of hyperaccumulator species are Ni hyperaccumulators (more than 75%, in 532 different species, as reported in Table 1), which is updated as of March 2020 by browsing the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/), which are found in a large number of naturally Ni-enriched serpentine soils worldwide (Reeves, 2003; Van der Ent et al., 2013). The main areas of distribution of Ni hyperaccumulator plants include the Mediterranean Region, especially with species in the genus *Alyssum* (Lombini, Dinelli, Ferrari, & Simoni, 1998; Reeves & Adigüzel, 2008; Shallari, Schwartz, Hasko, & Morel, 1998), the tropical serpentine soils in Cuba, where the Ni hyperaccumulator, *Phyllanthus pallidus*, was discovered (Reeves, Baker, Borhidi, & Berazaın, 1999), in New Caledonia with *Pycnandra acuminata* (formerly *Sebertia acuminata*), an endemic tree that accumulates 25% Ni in its latex (Jaffrè et al., 1976), and in Malaysia, where 16.9% Ni was found in the phloem sap exudates of *Phyllanthus balgooyi* (Van der Ent & Mulligan, 2015).

High Zn accumulation in *N. caerulescens* was first discovered in 1865, when plants growing in Zn-rich soils, near Germany–Belgium border (Sachs, 1865), were analysed. These plants contained at least 1% Zn in the dry leaves or 10% in the inorganic ash. Zn hyperaccumulator plants are less abundant than Ni hyperaccumulators and include, among Brassicaceae, *A. halleri* and different *Noccaea* species (Frey, Keller, Zierold, & Schulin, 2000; Zhao, Lombi, Breedon, & McGrath, 2000) and the Crassulaceae, *S. alfredii* (Yang, Long, Ni, & Fu, 2002). Since Zn, Cd and Pb share similar chemical properties, some Zn hyperaccumulators are also able to accumulate Cd and Pb (Krämer, 2010). Considering that Cd is a non-essential element and is highly toxic, Cd hyperaccumulation in plants is very rare and only a few species have been identified as Cd hyperaccumulators (Tian et al., 2017). This ability has been found mainly in *A. halleri* (Bert et al., 2003), *N. caerulescens* (Robinson et al., 1998) and *Noccaea praecox* (Vogel-Mikuš, Pongrac, Kump, Nečemer, & Regvar, 2006). Outside the Brassicaceae family, other Cd hyperaccumulator species are *Viola baoshanensis* (Violaceae; Liu, Shu, & Lan, 2004), *Salsola kali* (Chenopodiaceae; De La Rosa et al., 2004), *Solanum nigrum* (Solanaceae; Sun, Zhou, & Jin, 2006), *S. alfredii* (Crassulaceae; Deng et al., 2007) and *Phytolacca americana* (Phytolaccaceae; Liu, Peng, Wang, Lian, & Shen, 2009).

Manganese hyperaccumulation has been described in 42 species (Table 1, http://hyperaccumulators.smi.uq.edu.au/collection/, Reeves et al., 2017), endemic, primarily, in ultramafic soils (i.e. soils consisting predominantly in ferromagnesian silicate minerals, generally poor of essential mineral nutrients and characterized by high concentrations of phytotoxic elements, such as Ni, Co and Mn). The majority of manganese hyperaccumulators discovered, to date, have been found in New Caledonia and include *Virotia neurophylla* (Proteaceae) and *Maytenus founieri* (Celastraceae), which are able to accumulate 51,800 mg/kg Mn and 32,000 mg/kg Mn, respectively (Fernando et al., 2008; Jaffré, 1979). More recent studies demonstrated that

Gossia bidwillii (Myrtaceae) from Australia (Bidwell, Woodrow, Batianoff, & Sommer-Knusden, 2002) and *P. americana* (Phytolaccaceae) from China (Xue et al., 2004) are able to hyperaccumulate Mn in their leaves. Among Mn hyperaccumulating species found in China (summarized in Li et al., 2018), *P. americana* can accumulate up to 14,400 mg/kg Mn in its leaves when grown in a naturally contaminated Mn mine located in Xiangtan (Hunan Province, China). X-ray absorption spectroscopy assays, which are used to understand the mechanism of Mn hyperaccumulation in this species, demonstrated that *P. americana* has evolved some unique physiological mechanisms to cope with Mn hyperaccumulation in leaves, such as the Mn accumulation in epidermal cells (Xu et al., 2006) and complexation with oxalate (Xu, Shi, Chen, Chen, & Hu, 2009).

The hyperaccumulation of other metals, such as Pb, Se and As, has been reported. Pb hyperaccumulation is particularly rare and restricted to only a small number of species (Table 1). Indeed, Pb has low solubility in soil due to its ability to form complexes with natural organic matter and precipitate as carbonates, hydroxides and phosphates (Epstein et al., 1999). *Sesbania drummondii* is a perennial leguminous shrub from the southern coastal areas of the United States that hyperaccumulates Pb in shoots when grown in a Pb-contaminated nutrient solution (Sahi, Bryant, Sharma, & Singh, 2002). Another example of Pb hyperaccumulator is *Fagopyrum esculentum*, which accumulates a large amount of Pb in its leaves, stems and roots (Boonyapookana, Parkplan, Techapinyawat, DeLaune, & Jugsujinda, 2005).

Se hyperaccumulators occur mainly on naturally seleniferous soils in the Western United States and may accumulate Se in their tissues of up to 1.5% of dry weight (Pilon-Smits, 2019). Species hyperaccumulating Se are distributed across several genera of different families, among which are the Fabaceae, with the genus *Astragalus*, which harbour the most Se hyperaccumulators. The first report on Se hyperaccumulators concerned the Australian legume, *Neptunia amplexicaulis*, which is able to accumulate 4 mg/g of Se in leaves when grown on seleniferous soil (Peterson & Butler, 1967). Other Se hyperaccumulators have been found in the eudicot families, Amaranthaceae, Asteraceae, Brassicaceae, Orobanchaceae and Rubiaceae (White, 2016). Considering the wide distribution of Se hyperaccumulation across different families and genera, it is clear that this trait has evolved independently several times in the different taxa (Schiavon & Pilon-Smits, 2017).

Finally, only a few plant species have been characterized as As hyperaccumulators and most of these are fern species belonging to the Pteridaceae family (Ma et al., 2001; Meharg, 2003). There is speculation that the constitutive As hyperaccumulation trait has evolved in ferns as a consequence of the elevated concentrations of this element in the environments where they evolved (Meharg, 2003). The most studied As hyperaccumulator plant is *Pteris vittata*, identified in Central Florida soils contaminated with chromated copper arsenate (Ma et al., 2001). Apart from ferns, the only angiosperms displaying As hyperaccumulation are two Brassicaceae species, *Isatis cappadocica* and *Hesperis persica* (Karimi, Ghaderian, Maroofi, & Schat, 2009; Karimi, Ghaderian, Raab, Feldmann, & Meharg, 2009).

In brief, the wide distribution of hyperaccumulation over the plant phylogeny, as well as the variety of metals which it relates to, strongly indicates that this trait has evolved independently many times during plantae evolution. However, the extreme variability of accumulated metal ranges and plant behaviours in the different edaphic contexts, together with the occurrence of multi-metal hyperaccumulators, make it difficult to discriminate the effective origins of this phenomenon.

4 | GENETICS OF HYPERTOLERANCE AND HYPERACCUMULATION

Hyperaccumulation and hypertolerance are independent quantitative traits. Until now, genetic information on the molecular mechanisms underlying hyperaccumulation has mainly been restricted to Zn, Cd and Ni. This knowledge chiefly derives from studies of the Zn and Cd hyperaccumulators, *A. halleri* and *N. caerulescens*, because of their aforementioned close phylogenetic relationship with the model, *A. thaliana* (Krämer, 2010; Rascio & Navari-Izzo, 2011; Verbruggen et al., 2009). Information on the QTLs, controlling hypertolerance and hyperaccumulation, has been obtained by segregation experiments on inter- and intraspecific crosses and molecular genetics tools. In *A. halleri*, interspecific crosses with *A. lyrata* enabled the identification of one major QTL for Cd accumulation (Willems et al., 2010) and at least three QTLs for Zn hyperaccumulation (Filatov et al., 2007). In *N. caerulescens*, segregation analyses in F_2 population, derived from a cross between a plant growing in a contaminated site in Belgium and a plant growing on a similarly contaminated site in France, led to the identification of three QTLs, one accounting for Zn and Cd accumulation in shoots and two QTLs for both Zn and Cd accumulation in roots (Deniau et al., 2006).

The association of these QTL regions with the hyperaccumulation trait has allowed the identification of a great number of candidate genes involved in metal uptake, translocation and distribution, as the main determinants of metal hyperaccumulation. Moreover, comparative approaches, such as transcriptomics and proteomics, have also been applied to further identify the genes and proteins responsible for hyperaccumulation. Microarray analysis on *A. thaliana* chips has been applied to compare the transcriptomic profiles of the two Cd and Zn hyperaccumulator species, *N. caerulescens* and *A. halleri*, and the non-hyperaccumulator, *A. thaliana* (Becher, Talke, Krall, & Krämer, 2004; Van de Mortel et al., 2006; Weber, Harada, Vess, Roepenack-Lahaye, & Clemens, 2004). Transcriptomes derived from *N. caerulescens* ecotypes, with different metal uptake and tolerance levels, have also been compared (Plessl et al., 2010). Finally, the application of the modern technologies of next-generation sequencing to RNA sequencing has provided more precise information about gene expression in hyperaccumulator plants (Verbruggen, Hanikenne, & Clemens, 2013). These studies have demonstrated that the genetic determinants of heavy metal hyperaccumulation are constitutively overexpressed in hyperaccumulators compared to non-accumulator species. Considering that validation of transcriptomic data at the protein level is necessary, proteomic approaches have also been used to shed light on the modification of biological pathways that arises from changes in the gene transcription evolved by metal hyperaccumulator species (for a review, refer to DalCorso, Fasani, & Furini, 2013).

In recent years, sequencing projects have also involved hyperaccumulator species. However, advances in this field are still few and limited to those hyperaccumulators for which a closely related reference genome is available, such as *A. halleri* (Briskine et al., 2017). For example, whole-genome SNP comparisons between Polish metallicolous and non-metallicolous *A. halleri* populations identified 57 SNPs in 19 genes that were associated with edaphic conditions (Sailer et al., 2018). On the other hand, whole-genome scan analysis, comparing calamine and non-metallicolous populations of pseudo-metallophytes, *A. halleri* and *Arabidopsis arenosa*, highlighted limited evolutionary convergence among such species co-evolved in the same soils (Preite et al., 2019). In addition, some insight on candidate genes for metal tolerance and accumulation can be drawn from genome-wide association studies performed on non-accumulator crops and model species, such as *A. thaliana* (Chao et al., 2012), *Brassica napus* (Zhang et al., 2018, 2020), rice (Norton et al., 2014; Shrestha et al., 2018; Zhao et al., 2018) and maize (Zhao et al., 2017).

Overall, these different approaches have identified several genes that take part in metal transport and homeostasis, as well as genes that encode metal chelators, and are involved in the stress response. Among the determinants of metal tolerance and accumulation (for a comprehensive review, refer to Verbruggen et al., 2009, Rascio & Navari-Izzo, 2011), it is worth noting the fundamental role of metal transporters that belong to the HMA, MTP and NRAMP families. For example, plasma membrane transporter, HMA4, was found responsible for both Zn and Cd tolerance and accumulation (Courbot et al., 2007; Craciun et al., 2012; Willems et al., 2007, 2010), tonoplast transporters MTP1 (Dräger et al., 2004) and HMA3 (Liu et al., 2017; Ueno et al., 2011; Zhang et al., 2016) for Zn and Cd tolerance, respectively, as well as the vacuolar exporter, NRAMP1, for Cd accumulation (Milner et al., 2014). Ni hypertolerance and hyperaccumulation rely on histidine biosynthesis (Ingle, Mugford, Rees, Campbell, & Smith, 2005), as well as IREG/ferroportin transporters (Meier et al., 2018; Merlot et al., 2014).

5 | THE BRASSICACEAE FAMILY: A CASE STUDY FOR THE EVOLUTION OF THE HYPERACCUMULATION TRAIT

A special situation is represented by the Brassicaceae. This family has the highest occurrence of metal hyperaccumulator and hypertolerant species among the angiosperms, with 104 known hyperaccumulators within

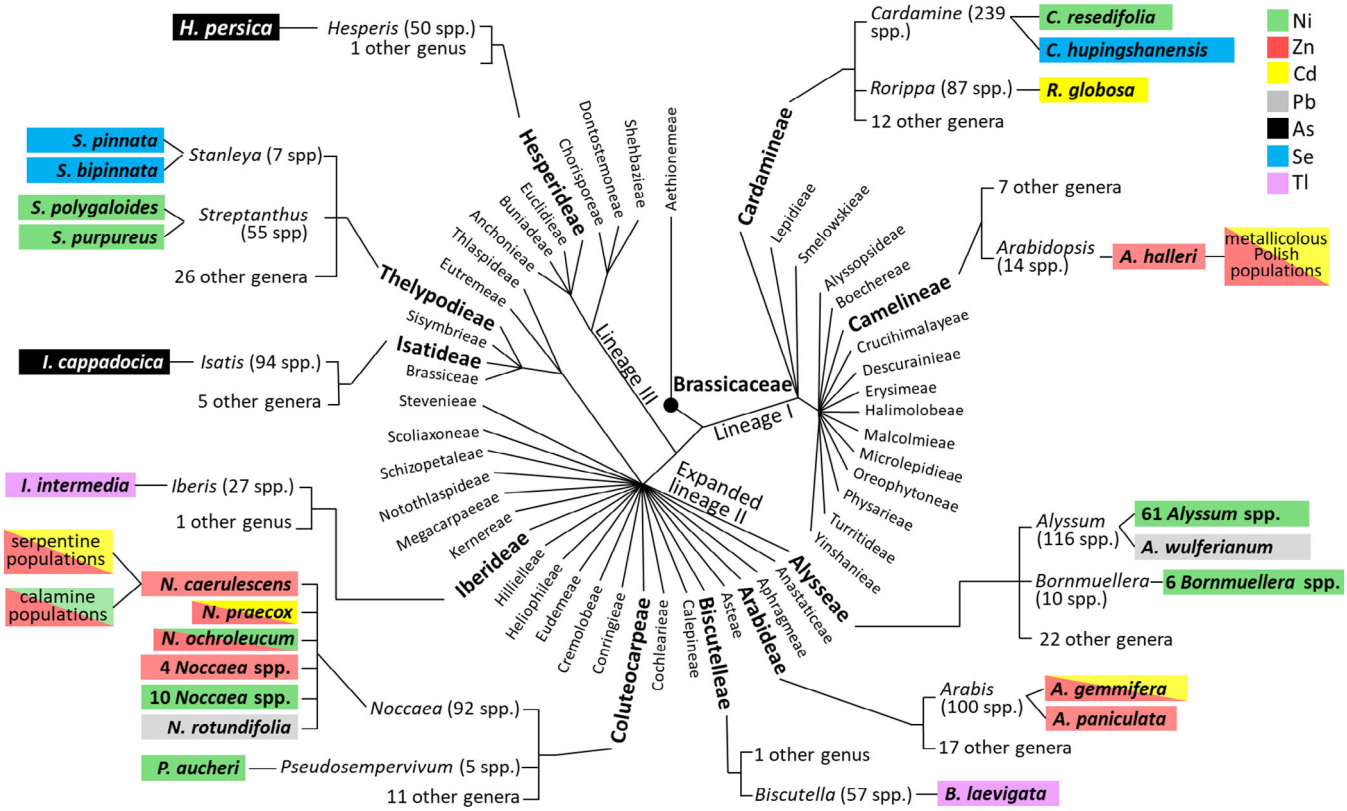

FIGURE 2 Graphical representation of the hyperaccumulator species in the Brassicaceae family: the trait evolved several times independently during the evolutionary history of the family. The Brassicaceae phylogenetic tree was constructed according to the BrassiBase database (https://brassibase.cos.uni-heidelberg.de/; Kiefer et al., 2014); tribes containing hyperaccumulators are in bold. Hyperaccumulators are highlighted with colours corresponding to the different metals accumulated, as described by the caption. Hyperaccumulator species are from the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/; Reeves et al., 2017); As hyperaccumulators were reported by Karimi, Ghaderian, Maroofi, and Schat (2009) and Karimi, Ghaderian, Raab, et al. (2009) [Colour figure can be viewed at wileyonlinelibrary.com]

an estimated family size of 3,710 species (Moray, Goolsby, & Bromham, 2016). As already mentioned, hyperaccumulation has evolved independently many times in the Brassicaceae lineage, as elucidated by Figure 2 (for a more detailed representation of evolution of Se and Ni hyperaccumulation in the *Stanleya* genus and in the Alysseaea tribe, respectively, refer to Cappa et al., 2015; Cecchi et al., 2010). Together with the Euphorbiaceae family, Brassicaceae contributes approximately 25% of known Ni hyperaccumulators, as well as most of the Zn and Cd hyperaccumulators. As, Se and Pb hyperaccumulation has also been reported in this family (Krämer, 2010). Brassicaceae makes excellent specimens for understanding the evolution of the metal hypertolerance/hyperaccumulation trait. In particular, member species cover a variety of different behaviours related to metal tolerance and accumulation, ranging from excluders and metal indicators to hypertolerant and hyperaccumulator species.

The characterization of hypertolerance and hyperaccumulation evolution in the Brassicaceae lineage has been arousing increasing interest, particularly in the last decade. Research studies have recently supported the existence of multiple onsets of the trait, affecting both large and small groups, from genus subsections to individual populations, painting an extremely complex picture (Figure 2). For instance, an interesting system for Ni hyperaccumulation has been found in the *Alyssum* genus, including 50 Ni hyperaccumulators and other related non-accumulators (Cecchi et al., 2010). Specifically, hyperaccumulation has evolved only in the monophyletic lineage, *Alyssum*, section, *Odontarrhena*, although some species from different sections display various levels of Ni tolerance. However, not all *Odontarrhena* species are serpentinophytes. The presence of both hyperaccumulators and non-accumulators in each phylogenetic branching of this section indicates that, in this context, hyperaccumulation has multiple evolutionary origins, as well as possible losses of the trait (Cecchi et al., 2010). A similarly complex situation emerged with regards to Se hypertolerance/hyperaccumulation in the *Stanleya* genus. Although tolerance is widespread in the genus and correlates with Se storage in organic forms, hyperaccumulation is restricted to the *Stanleya pinnata/bipinnata* clade, supporting the hypothesis that Se tolerance precedes, and is necessary for, hyperaccumulation (Cappa et al., 2015). However, also in this phylogenetic context, the presence of non-tolerant and non-accumulator varieties of *S. pinnata* indicates that the trait may have been lost in non-metallicolous environments (Cappa et al., 2015).

Adding to this complexity, pseudo-metallophyte species, such as *A. halleri* and *N. caerulescens*, display wide levels of variability in tolerance and accumulation between different populations. Indeed, all known *A. halleri* and most *N. caerulescens* populations are hypertolerant to Zn and show consistent accumulation of this metal, up to the point of hyperaccumulation, when grown in Zn-rich substrates (Assunção et al., 2003; Babst-Kostecka et al., 2018; Stein et al., 2017). However, their behaviour in relation to other metals, such as Cd, Pb and Ni, is extremely variable and depends on the edaphic context in which they evolved (Assunção et al., 2003; Stein et al., 2017). For instance, it was suggested that Zn hypertolerance and hyperaccumulation in *A. halleri* evolved concurrently with speciation from its sister species, *A. lyrata* (Roux et al., 2011), and predating adaptation to metallicolous and non-metallicolous soils (Meyer et al., 2016). With regard to this trait, some variability is also appreciable in the different populations, suggesting that evolution is progressing toward higher Zn hyperaccumulation in non-metallicolous sites and lower in metallicolous ones (Babst-Kostecka et al., 2018). On the other hand, Cd hypertolerance in *A. halleri* is strongly associated with the edaphic context, with metallicolous populations proving to be significantly more tolerant than non-metallicolous ones (Meyer et al., 2015). Interestingly, the evolution of Cd hyperaccumulation is likely to have followed the separation of *A. halleri* into distinct phylogeographic groups, as proposed by Pauwels et al. (2012), as it is notably lacking in the southeastern unit (Stein et al., 2017). In general, metallicolous populations have adapted from spatially close, non-metallicolous ones, colonizing metal-rich environments (Pauwels et al., 2005).

As for *N. caerulescens*, the variability of behaviour among the different populations toward metals is even more noticeable, although the investigation into the evolutionary aspect of the phenomenon is still in its early stages. Indeed, significant differences in tolerance and accumulation of Zn, Cd and Ni have been observed in *N. caerulescens* populations deriving from non-metallicolous, calamine and serpentine soils (Assunção et al., 2003; Escarré, Lefèbvre, Frérot, Mahieu, & Noret, 2013; Gonneau, Genevois, Frérot, Sirguey, & Sterckeman, 2014; Roosens et al., 2003). In particular, the evidence regarding root-to-shoot translocation, homeostasis of other elements and fitness-related traits suggests that populations from different soil types have evolved different mechanisms for metal tolerance and accumulation (Assunção et al., 2003; Gonneau et al., 2014). Despite the extreme variability of phenotype, the genomic structure of *N. caerulescens* is apparently stable at the intra-specific level, indicating that these differences in behaviour are not associated with large chromosomal rearrangements (Mandáková, Singh, Krämer, & Lysak, 2015).

However, the same genomic analysis highlighted alterations in chromosomal structure compared to the non-accumulator, *A. thaliana*. In particular, several pericentric inversions relocated metal homeostasis genes in distal, euchromatic positions, leading to higher expression and contributing to the overall evolution of species-wide metal tolerance/accumulation (Mandáková et al., 2015). As with *A. halleri*, the colonization of different metallicolous environments follows speciation and separation into phylogeographic groups, indicating that specific adaptive evolution occurred independently and locally several times (Gonneau et al., 2017).

6 | WHY DID HYPERTOLERANCE AND HYPERACCUMULATION EVOLVE?

All organisms must cope with, and adapt to, the environment in which they live and particular efforts are made by organisms that are not able to quickly escape adverse environmental conditions.

Since the discovery of hypertolerance and hyperaccumulation, the interest of ecologists has been attracted by the very high natural selection and rapid evolution of these traits (Macnair, 1997).

Substantial research in this field suggests that metal tolerance on facultative metallophytes could evolve rapidly following exposure to a high level of heavy metals (Al-Hiyaly, McNeilly, & Bradshaw, 1988). In addition, geographically distant conspecific populations could have evolved independently and so constitute a polyphyletic group (Schat, Vooijs, & Kuiper, 1996). For the vast majority of these species, heavy metal tolerance is achieved by a metal excluder strategy.

Recent experiments have highlighted that, at least under controlled experimental conditions, elevated concentrations of heavy metals, such as Zn, which mostly affect survival and the reproductive ability of plants, can reasonably be considered as a selective agent, inducing plants, to evolve metal tolerance (Nowak et al., 2018). Indeed, within a few generations, non-metallicolous individuals of *N. caerulescens* exposed to increased Zn concentrations developed physiological traits associated with Zn tolerance (Nowak et al., 2018).

The mechanisms underlying such metal–plant interactions still require investigation, but an alternative hypothesis involves a metal-induced genome methylation, which would be vertically inherited as a form of trans-generational stress adaptation. In any event, firm evidence for the inheritance of changes in the methylome, induced by environmental stresses, is still lacking (Weinhold, 2018). As described in previous paragraphs, hyperaccumulation is a relatively uncommon trait that is believed to have arisen several times independently (Broadley, White, Hammond, Zelke, & Lux, 2007) and has been acquired by a relatively small number of plant families. Several different theories have been postulated in the past decades in an attempt to explain the evolutionary origin of hyperaccumulation (Figure 3). The pioneer in the field is a publication by Boyd and Martens (1992), which highlighted the putative reasons underlying the evolution of this trait. One hypothesis involves allelopathy (i.e. the phenomenon by which a plant can influence the germination, growth and reproduction of other plants). In the case of hyperaccumulating plants, it has been proposed that when element-enriched dead plant material is cycled back into the soil, the release of toxic elements and their accumulation in the top layer of soil can negatively affect competitors (Jaffe, Ketterer, & Shuster, 2018). There is little consensus on such elemental allelopathy. Indeed, the toxic metals accumulated in leaves are absorbed from the contaminated soil in which plants grow, so local plants are usually tolerant of the excess elements. Evidence for naturally occurring elemental allelopathy is, therefore, limited (Jaffe

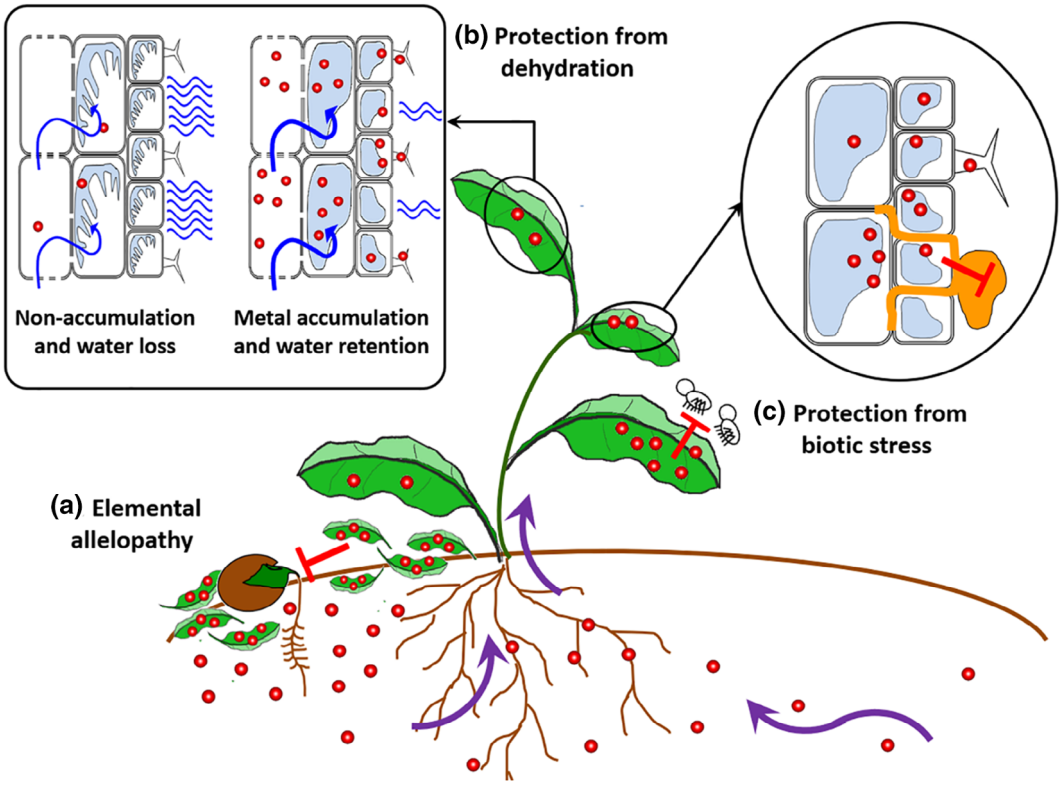

FIGURE 3 Graphics summarizing the main theories tentatively explaining hyperaccumulation evolution. (a) Elemental allelopathy has been postulated as an enrichment of metal and metalloids (depicted by red dots) in the surroundings of the hyperaccumulator plants, directly impairing germination and growth of metal-sensitive local plants. Metal 'flow' from the soil towards the site of deposition of metal-enriched dead plant material is represented by violet arrows. (b) Even if experimentally controversial, protective effects of heavy metal accumulation have been proposed against drought stress. Both main mechanisms attributed to metal ions, which can reduce cuticular transpiration (represented as blue waves) or enhance cell osmolarity in a way similar to compatible solutes or inorganic ions driving water accumulation into the vacuole (blue arrows), are depicted in the figure. (c) Elemental defence hypothesis has been also reported, showing the mechanisms of effective inhibition of herbivores (schematized as insects) and fungal pathogens (spore and intercellular hyphae growth are highlighted in orange) [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2018). However, a few promising experiments have been conducted on elemental allelopathy, generated by the metalloids, As and Se. The results obtained suggest that, in the case of the As hyperaccumulating fern, *P. vittata*, its ability to concentrate As in the surrounding soil to a concentration that is nearly twice as high as that of nearby sites could actually provide a competitive advantage over other local plants (Jaffe et al., 2018). Similar enrichment effects have been described in *Astragalus bisulcatus* and *S. pinnata*. Indeed, like *P. vittata*, these Se hyperaccumulators are also able to increase Se concentration in their surrounding soil, directly impairing the growth of local Se-sensitive plant species (El Mehdawi, Quinn, & Pilon-Smits, 2011).

A second, rather debatable hypothesis for the evolution of the hyperaccumulation trait points to an induced protection from drought stress (Boyd & Martens, 1992). This theory is based on the consideration that naturally metal-enriched soils, such as serpentine and seleniferous lands, due to their poor structure, high porosity and high hydraulic conductivity, are usually prone to water percolation and drought (Bhatia et al., 2005). Furthermore, a positive correlation between drought tolerance and the ability to survive on serpentine substrates has been reported (Hughes, Bachmann, Smirnoff, & Macnair, 2001). A variety of mechanisms have been proposed to describe the effect of metals on drought resistance. For instance, metals, being localized in the epidermis of leaves, may reduce cuticular transpiration or enhance cell osmolarity in a similar way to compatible solutes or inorganic ions, which accumulate on water stress in order to raise the osmotic potential of cells (Whiting, Neumann, & Baker, 2003). The results obtained from experiments are controversial, some pointing to a lack of evidence for the drought tolerance hypothesis (e.g. for Ni in *Alyssum murale*, Zn in *N. caerulescens* and Se in *A. bisulcatus*; Statwick, Williams, & Sher, 2017; Whiting et al., 2003), while others are consistent with an osmoregulatory role by which Ni counteracts drought stress, as in the Australian Ni hyperaccumulator, *Stackhousia tryonii* (Bhatia et al., 2005). However, an alternative interpretation of this particular case is that Ni could be an accidental actor in adjusting drought response, since it is absorbed and not directly metabolized by the plant cells, offering a passive and physical action as an osmoticum (Bhatia et al., 2005). In this view, hyperaccumulation is far from being a direct, adaptive strategy to provide drought protection.

The third and most intriguing theory of the evolution of metal hyperaccumulation, usually referred to as the 'elemental defence hypothesis' (or 'defensive enhancement hypothesis'; Boyd, 2012), describes this mechanism as an effective defence against herbivores and pathogens (Boyd, 2007). To counteract attacks by pathogens, plants have developed a variety of chemical defences, featuring specifically synthetized organic compounds (e.g. glucosinolates, tannins and other molecules) or inorganic moieties that are obtained (or metabolized) from the growth substrate. This latter strategy has been observed in plants with enhanced silicification, that is, the accumulation of silica in the plant tissues (in the form of silicic acid), and with fluoroacetate synthesis and accumulation (Exley, 2015; Leong, Khan, Davis, Denman, & McSweeney, 2017). The protective effect of hyperaccumulation is exhibited as both deterrence and toxicity towards the pathogen. Evidence for this defence has been produced experimentally in most hyperaccumulated metal moieties (Boyd, 2007). It is worth noting that the pathogen attack itself is responsible for the induction of mechanisms underlying hyperaccumulation and hypertolerance (i.e. the upregulation of *HMA4* transporter gene in *N. caerulescens* on treatment with the fungus, *Alternaria brassicicola*, Gallego, Martos, Cabot, Barceló, & Poschenrieder, 2017). The effectiveness of metal defence depends on the kind of pathogen considered. Examples of the growth inhibition of herbivore arthropods, as well as a reduction in the growth and infection magnitude of bacterial and fungal pathogens, have been reported, while virus infections seem to be rather insensitive to metal hyperaccumulation in the host plant (Davis, Murphy, & Boyd, 2001). There are many references that describe the deterrence effects of metals and metalloids on phytopathogenic bacteria and fungi, herbivore slugs and insects, and even small mammals, such as the prairie dog *Cynomys ludovicianus* (Boyd, 2007; Quinn, Freeman, Galeas, Klamper, & Pilon-Smits, 2008). In particular, the great variety of feeding mechanisms in herbivores (e.g. xylem or phloem feeders, leaf chewers, cell disruptors) directly influences the effectiveness of the elemental defence (Hörger, Fones, & Preston, 2013). In support of the defensive role of hyperaccumulated metals, it appears that the element is often concentrated in those organs and tissues that are the prime site of infection or the most susceptible to herbivores (Freeman, Quinn, Marcus, Fakra, & Pilon-Smits, 2006). For instance, metals accumulated in the apoplast of *N. caerulescens*, in which the pathogen *Pseudomonas syringae* pv. *maculicola* multiplies, reach values that exceed the concentrations required to inhibit bacterial growth, at least in vitro (Fones & Preston, 2013).

Heavy metals and metalloids accumulated in plant tissues can display their effects against biotic stress by means of a range of mechanisms. Firstly, their accumulation can be directly toxic for herbivores and pathogens. Moreover, considering that plant responses to both metal toxicity and pathogens share part of the signalling pathways (e.g. the formation of reactive oxygen species) (Overmyer, Brosche, & Kangasjarvi, 2003), metals can alter the cross-signalling between abiotic and biotic stress, as well as those pathways activated in response to biotic attacks. Furthermore, high metal concentrations can be effective in enhancing alternative biotic stress signalling pathways, offering indirect protection against parasite attack (Llugany, Martin, Barceló, & Poschenrieder, 2013). For example, in Zn and Ni hyperaccumulating *N. caerulescens*, the glutathione concentration is constitutively elevated, leading to enhanced tolerance to metal-induced oxidative stress. This is a consequence of the constitutively high levels of salicylic acid and its derivatives that are observed in these plants (Freeman et al., 2006). Salicylic acid is well known to be involved in plant responses to pathogens, the hypersensitive response and systemic acquired resistance (Shah, 2003). Interestingly, the absence of Ni in the growth medium makes the hyperaccumulator, *Noccaea goesingense*, more susceptible to powdery mildew (*Erysiphe cruciferarum*). This is because, under this condition, no increase in salicylic acid synthesis could be detected and the defense responses induced by this hormone were suppressed. Conversely, the accumulation of Ni in *N. goesingense* enhances resistance to *E. cruciferarum*,

suggesting that Ni hyperaccumulation may compensate for the loss of the salicylic acid-dependent pathogen response of these plants (Freeman, Garcia, Kim, Hopf, & Salt, 2005). Similarly, susceptibility to *E. cruciferarum* in the Zn/Cd hyperaccumulator, *N. praecox*, under low metal treatment has also been attributed to defective salicylic acid synthesis (Llugany et al., 2013).

When the accumulation concerns essential metals, such as Zn, which are involved in both pathogen virulence strategies and the host plant's defense mechanisms, the situation is more complicated. This occurs in the pathosystem, *N. caerulescens*, and the necrotrophic fungus, *Alternaria brassicicola* (Gallego et al., 2017). In this case, the evolution of hyperaccumulation and its effect on both the resistance mechanisms and the compatibility of a parasite–host relationship may vary greatly, depending on the species ecotype considered, the metal(s) hyperaccumulated and the pathogen targeted, and no straightforward generalization is possible.

Lastly, it must be considered that the interactions between hyperaccumulating plants and their pathogen are continuously evolving and the parasites involved can acquire resistance to the heavy metals accumulated by their hosts (Boyd, 2012). A specific example is given by the so-called 'high-Ni insects' (Boyd, 2009). The grasshopper, *Stenoscepa* sp., is preferentially attracted by Ni-hyperaccumulating plants, on which it feeds without showing any symptoms of toxicity (Augustyniak et al., 2008), and the hemipteran, *Melanotrichus boydi*, accumulates Ni, by feeding on the hyperaccumulator, *Streptanthus polygaloides*, converting the metal accumulated into a protection mechanism against predation (Boyd & Wall, 2001). Moreover, a strain of the moth, *Plutella xylostella*, has adapted to feed on the Se hyperaccumulator, *S. pinnata*. Tolerant moth-sequestered Se has been found in the hindgut or in secreted deposits on the exterior of the abdomen, suggesting that, as with Ni, Se can also be accumulated by insects and used as a defense against predation (Freeman et al., 2006). Pathogenic bacteria also seem to have acquired enhanced tolerance to metals, due to their evolutionary relationship with hyperaccumulators. Many studies have demonstrated that endophytic bacteria, isolated from Ni and Zn hyperaccumulators, exhibit higher metal tolerance than related bacteria isolated from a non-accumulating plant (Fones et al., 2010).

There are at least three remarkable corollaries to the accumulation of metals. Firstly, hyperaccumulating plants may need more basal metal than non-accumulator plants (Fones & Preston, 2013). As previously mentioned, metal hyperaccumulator species are at a competitive disadvantage to non-metalliferous soils, when compared to neighbouring non-accumulating species. For instance, increased mortality and reduced seed production have been observed in hyperaccumulating ecotypes of *N. caerulescens* grown on 'normal' soils, while other ecotypes underwent a 75% increase in biomass when supplied with Cd (Halimaa et al., 2014; Roosens et al., 2003). Essential metals, such as Zn, Mn or Ni play a role in a variety of processes, such as reactive oxygen species metabolism, the activity of dismutase enzymes (such as Mn-superoxide dismutase) and the metabolism of urea and glutamine synthesis (Cakmak, 2000; Witte, 2011; Yruela, 2013). Their disturbance due to a depletion of essential metals may contribute to the poor health of hyperaccumulating plants on non-metalliferous soils.

Secondly, hyperaccumulating plants are usually able to accumulate more metals, if available on their growth substrate, and the different elements may interact in a synergic manner with each other and with the organic defence compounds, lowering the concentrations at which they become effective against the biotic attack. This occurrence has been named the *joint-effects hypothesis*, advanced by Boyd (2012).

Thirdly, the chemical defence apparatus to combat pathogens seems to be diminished by metal accumulation, a phenomenon described in the *trade-off hypothesis* (Farinati et al., 2009; Fones & Preston, 2013). Hyperaccumulation and the derived metabolic and genetic adaptations that enable the accumulation of high levels of potentially toxic metals (such as increased antioxidant production, enhanced expression of metal uptake transporters, metal translocation and accumulation in the vacuole) require a lot of energy and hyperaccumulator plants may compensate for this by reducing the investment of energy in the disease resistance equipment (Maestri et al., 2010). Evidence has been reported from a proteomic approach on *A. halleri* shoots, where treatment with Cd and Zn was able to downregulate the signalling networks that mediate plant defense responses against herbivores, insects and pathogen attacks (Farinati et al., 2009). A trade-off between Zn and glucosinolates in shoots of *N. caerulescens* and between Ni and glucosinolates in *Streptanthus polygaloides* has also been described (Tolrà, Poschenrieder, Alonso, Barceló, & Barceló, 2001). Interestingly, in the latter case, this trade-off between organic and Ni-based defense is likely to be constitutive and not affected by edaphic conditions (Davis & Boyd, 2000).

In conclusion, the evolution of hyperaccumulating plants must be considered with regard to the context where natural populations have evolved, in which a variety of joint effects and trade-offs between different forms of defense are possible, constantly remodelling and adjusting to the environment, and, therefore, steering the genome and epigenome (see next paragraphs) of plants towards evolutionary success.

7 | HOW DID HYPERTOLERANCE AND HYPERACCUMULATION EVOLVE?

As discussed, a range of genetic evidence indicates that the evolution of both hypertolerance and hyperaccumulation includes (a) macro-scale evolutionary events that resulted in species-wide tolerant/accumulating backgrounds, and (b) local adaptive processes in different edaphic environments, leading to specific strategies and various degrees of hypertolerance and hyperaccumulation.

7.1 | Macro-scale evolution of species-wide metal tolerance and accumulation

Several specific traits have been identified that distinguish hyperaccumulator species from closely related, non-accumulator ones, outlined in Figure 1. For instance, upregulation of histidine biosynthesis

was correlated with Ni accumulation in the genus, *Alyssum* (Ingle et al., 2005). Se accumulation was associated with its assimilation in organic forms in *Stanleya* spp. (Cappa et al., 2015). Another feature characteristic of some hyperaccumulator species is the higher copy number and enhanced *cis*-regulation of metal transporters (Hanikenne et al., 2008; Ó Lochlainn et al., 2011; Shahzad et al., 2010). Mutations in the coding sequence of metal transport genes or different splicing processes have also been reported: for example, alternative splicing of the *NgMTP1* transcript leads to the modification of metal selectivity toward Ni in the Ni hyperaccumulator, *N. goesingense* (Persans, Nieman, & Salt, 2001). Likewise, sequence divergence in the coding sequence of ATP sulfurylase, *ATPS2*, in Se hyperaccumulator, *S. pinnata*, and related non-accumulator, *Stanleya elata*, leads to the loss of plastidic localization in the former and an increased accumulation in the cytosol (Jiang et al., 2018). However, clear evidence linking specific evolutionary changes, with the insurgence of hypertolerance and hyperaccumulation at the species levels, is still scarce.

The most apparent example of species-wide evolution of metal tolerance and accumulation is in *A. halleri*. This species has diverged from its close relative, *A. lyrata*, in relatively recent times, at the same time, as the first duplication of the *AhHMA4* gene, leading to major adaptive changes in terms of metal homeostasis, tolerance and accumulation (Hanikenne et al., 2008; Roux et al., 2011). Interestingly, *AhHMA4* was found to colocalize with the major QTLs for Zn and Cd hypertolerance and hyperaccumulation in metallicolous populations (Courbot et al., 2007; Willems et al., 2007, 2010) and with the single QTL for Zn and Cd tolerance identified in the non-metallicolous population, SK2 (Meyer et al., 2016). In addition, triplication of *AhHMA4* is retained in the entire *A. halleri* species (including both the European subsp. *halleri* and the Japanese subsp. *gemmifera*), further supporting the role of expansion in the *HMA4* copy number in the emergence of hypertolerance and hyperaccumulation and in the speciation of *A. halleri* (Briskine et al., 2017; Hanikenne et al., 2008, 2013; Meyer et al., 2016). It is clear that *AhHMA4* alone is not able to completely determine the hypertolerance and hyperaccumulation phenotypes. Indeed, in the metallicolous *A. halleri* population of Auby, the QTL, including *AhHMA4*, was found responsible for only 12% of the phenotypic variance in Zn tolerance and 43% in Cd tolerance (Courbot et al., 2007; Willems et al., 2007), indicating that other genes are involved in the phenomenon. However, given the previous evidence, *AhHMA4* duplication is likely to be the evolutionary event that produced the accumulating background of *A. halleri*, providing the genetic basis for the further adaptation of metallicolous populations (Meyer et al., 2016). Interestingly, genetic diversity in the promoter regions of the three copies of *AhHMA4* is extremely low among the different metallicolous and non-metallicolous *A. halleri* populations, both from the *halleri* and *gemmifera* subspecies. In the light of this, great selective pressure must have driven positive selection in the regulatory regions during the evolution of *A. halleri*, leading to increased expression levels of each *AhHMA4* copy, in addition to copy number expansion (Hanikenne et al., 2013).

The evolutionary history of *N. caerulescens* was marked by a similar phenomenon. A common tolerant, accumulating background also developed in the species early in its evolution, followed by adaptation to specific environments (Gonneau et al., 2017; Mandáková et al., 2015). Specifically, significant chromosomal rearrangements were found in *N. caerulescens* when compared to *A. thaliana*, involving several genes associated with metal homeostasis. Five rearrangements out of seven were retained in two close relatives (*Noccaea jankae* and *Raparia bulbosa*) that were able to accumulate metals at moderate levels, indicating that evolution toward metal hyperaccumulation began before *N. caerulescens* speciation and has progressed over its evolutionary history (Mandáková et al., 2015). Most interestingly, the region that includes *NcHMA4* is among the genomic portions subject to pericentric inversions, placing this gene in a distal, euchromatic section of the chromosome. This change results in increased expression but may also produce higher recombination rates in the *NcHMA4* gene, enabling further evolution of the hypertolerance/hyperaccumulation trait (Mandáková et al., 2015).

7.2 | Local adaptive processes in different edaphic environments

Although a species-wide predisposition to metal tolerance and accumulation is often present, intraspecific genetic and phenotypic variability is generally significant in the hyperaccumulator species, suggesting that adaptation to metallicolous environments is a constantly evolving trait (Adamidis, Aloupi, Kazakou, & Dimitrakopoulos, 2014; Assunção et al., 2003; Babst-Kostecka, Parisod, Godé, Vollenweider, & Pauwels, 2014; El Mehdawi, Paschke, & Pilon-Smits, 2015; Meier et al., 2018; Schvartzman et al., 2018; Sobczyk, Smith, Pollard, & Filatov, 2017; Stein et al., 2017). This diversity is clearly related to the composition of native soils (Assunção et al., 2003; Babst-Kostecka et al., 2014; Meyer et al., 2015; Preite et al., 2019), as well as other environmental factors (Frérot et al., 2018). However, geographical separation and, occasionally, long-distance anthropic dispersal also played significant roles in genetic differentiation since metallicolous populations have often adapted to extreme environments, starting from spatially close, non-metallicolous populations with a tolerant/accumulating background (Gonneau et al., 2017; Pauwels et al., 2005; Preite et al., 2019). On testing 12 highly polymorphic microsatellite *loci* on metallicolous and non-metallicolous populations of *Commelina communis* (a pseudo-metallophyte that grows on both cupriferous habitats and the surrounding non-metalliferous areas), geographically distant metallicolous populations displayed greater genetic similarity to their nearby non-metallicolous ecotypes than to each other, whereas edaphic conditions had little effect on genetic variability (Li et al., 2016). It is, therefore, likely that the widely distributed *C. communis* metallicolous population evolved independently, rather than from a single tolerant ancestral population (Ye et al., 2012). Given this complex scenario, hypertolerance and hyperaccumulation in metallicolous populations of pseudo-metallophytes have generally evolved independently multiple times, which probably led to different strategies in geographically distant populations of the same species.

Among the processes involved in adapting to metallicolous soils, evolutionary dynamics mainly influence metal translocation and

compartmentalization. For instance, sequence differentiation was found in the genes, *NRAMP4* and *IREG1*, coding for metal ion transporters, in serpentine and non-metallicolous populations of *Alyssum serpyllifolium*, which suggest the evolution in the former of specific strategies for trace element homeostasis (Sobczyk et al., 2017). Similarly, genetic variation was found associated with soil metal concentration in an analysis comparing metallicolous and non-metallicolous populations of *A. halleri* from Poland. Considering the genes linked to adaptation to metallicolous soils, there was a significant enrichment of ion transport and stress response pathways (Sailer et al., 2018). On the other hand, the aforementioned *A. halleri* metallicolous populations, I16 and PL22, which belong to different phylogeographic units, have evolved distinct strategies to cope with Zn and Cd excess, involving the modulation of very different sets of genes for metal uptake and storage, mineral homeostasis and stress response (Corso et al., 2018; Schvartzman et al., 2018). Flavonoid-related genes, which play a role in metal and ROS detoxification and are constitutively highly expressed in PL22, with a consequently high accumulation of flavonoids in shoots (Corso et al., 2018), is a clear example of this. The evidence that the genetic unit was the main driving force by far of the observed variation of gene expression, whereas metal treatment had only a marginal effect on this variance, further supports the hypothesis of the independent adaptation of geographically distant metallicolous populations to extreme edaphic conditions, starting from non-metallicolous populations (Corso et al., 2018; Schvartzman et al., 2018).

From a molecular point of view, genetic events, leading to adaptation to metallicolous environments, are analogous to those observed in the evolution of species-wide metal tolerance/accumulation, as reported in Figure 4. For example, sequence divergence in the *S. alfredii HMA3* gene, encoding a tonoplast transporter, leads to significantly higher transcription levels, preferential shoot expression and a marked selectivity for Cd in a hyperaccumulator ecotype compared to a non-accumulating one, displaying lower, equally distributed expression and Zn and Cd specificity (Zhang et al., 2016).

Duplication events have also occurred with metal transporter genes, differentiating populations in different edaphic conditions. For example, *HMA4* copy number, the expansion of which was fundamental in *A. halleri* speciation, was not fixed in *N. caerulescens*. Indeed, calamine populations had more copies of this gene than serpentine ones, and the *NcHMA4* number is directly correlated with the efficiency of Cd uptake and translocation (Craciun et al., 2012).

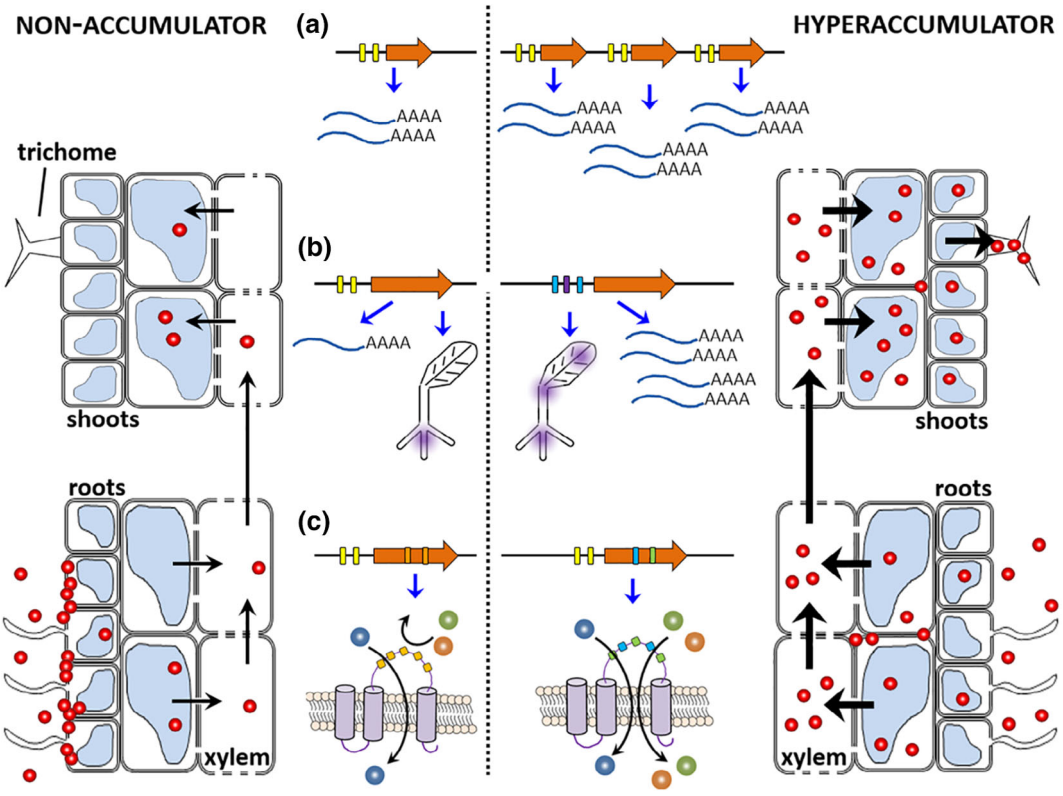

FIGURE 4 Main mechanisms involved in acquisition of heavy metals and metalloids hyperaccumulation ability in plants (on the right side) showed in contrast with non-accumulating plants (on the left side). (a) Events of gene (symbolized as orange arrows) duplication results in enhanced expression of genes involved in the process of metal transport (the latter exemplified as black arrows). (b) Changes in the promoter sequences (represented as coloured squares upstream the gene—orange arrows) have enhanced the expression of important hyperaccumulation determinants, and modified the expression localization through the plant body and tissues, allowing accumulation of metals in specific detoxification districts (e.g. the subepidermal leaf cells or the trichomes). (c) Changes in the coding sequence can modify the splicing site of introns or modify the sites responsible for metal specificity, being directly involved in the evolution of transport capacity of metal transporters (Metal and metalloids are indicated by red dots. Generic metal ions are shown as blue, green and orange dots) [Colour figure can be viewed at wileyonlinelibrary.com]

A similar phenomenon was observed for *A. halleri MTP1*. This gene, coding for a tonoplast Zn transporter, is associated with QTLs for Zn tolerance and is present in multiple copies in metallicolous populations (Dräger et al., 2004; Shahzad et al., 2010; Willems et al., 2007). On the other hand, the non-metallicolous population, SK2, has only one copy of *AhMTP1* that is not associated with metal tolerance, QTLs (Meyer et al., 2016). In addition, *AhMTP1* in non-metallicolous population, SK2, is expressed at comparable levels to those in non-tolerant *A. lyrata* (Meyer et al., 2016), whereas expression of each *AhMTP1* copy in the metallicolous populations of Auby and I16 are significantly higher (Fasani et al., 2017; Shahzad et al., 2010). Interestingly, the enhancement of *cis*-regulation, as well as the acquisition of new domains leading to *MTP1* expression in trichomes, was acquired independently by both metallicolous populations, which belonged to different phylogeographic units, as suggested by the divergence in the *AhMTP1* genomic sequences (Fasani et al., 2017; Shahzad et al., 2010). This evidence supports the hypothesis that convergent evolution has occurred in some cases during the adaptive processes for Zn hypertolerance.

8 | CONCLUSIONS

In conclusion, metal hypertolerance/hyperaccumulation is a very complex, continuous, evolutionary phenomenon, involving a variety of processes, ranging from metal uptake and translocation to signalling and stress response. The range of levels on which it developed independently, as well as the genomic and epigenomic flexibility enabling quick adaptation to extreme edaphic contexts, make this trait a unique model for the study of evolution and adaptation to stresses. From this perspective, metal hypertolerance and hyperaccumulation make up a varied scenario that involves not only plant interaction with different soil types but also complex integration with ecological niches and the co-evolution of associated pathogens.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Anna Manara, Elisa Fasani, Antonella Furini and Giovanni DalCorso have made substantial contributions to conception and design of the manuscript, have been involved in drafting the manuscript and revising it critically for important intellectual content and have given final approval of the version to be published.

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